THE ISOLATION AND IDENTIFICATION OF THE ANTHOCYANIN IN THE LEAVES OF *OXALIS ORTGIESSI*

An abstract of a Thesis by
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Drake University

The problem. The purpose of the present investigation was to isolate and identify the anthocyanin in the leaves of *Oxalis ortgiessi*.

Procedure. The leaves were extracted in 1% HCl in methanol for 24 hours. This extract was concentrated *in vacuo* and then streaked on 3 mm Whatman chromatography paper. The chromatogram was developed by ascending chromatography in 1% HCl. After separation the band was cut out and eluted in 0.01% HCl in methanol. These solutions were reduced in volume under vacuum and absorption spectrum determined. The purified elute was spotted on Whatman No. 1 and the Rf values of the pigment determined in four different solvent systems. Acid and alkaline hydrolysis was carried out to identify the aglycone, glycosides and organic acid.

Findings. The anthocyanin of the leaves of *Oxalis ortgiessi* was identified as malvidin-3-rhamnoglucoside-5-glucoside from the Rf values, spectral maxima and color observations.

Conclusion. The anthocyanin of the leaves of *Oxalis ortgiessi* probably could not be used to aid in taxonomic work because the species is an ornamental plant developed for the color of the leaves.

Recommendations. Further studies of the flower pigments of *Oxalis ortgiessi* is needed to see if there are any relationships between leaf and flower pigments. Isolation and identification of pigments in other species in the Oxalidaceae family needs to be done to aid in taxonomic study of this group to show relationships.

THE ISOLATION AND IDENTIFICATION OF THE ANTHOCYANIN IN THE LEAVES OF *OXALIS ORTGIESII*

A Thesis

Presented to

The School of Graduate Studies

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In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

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by

Charles Louis Lehman

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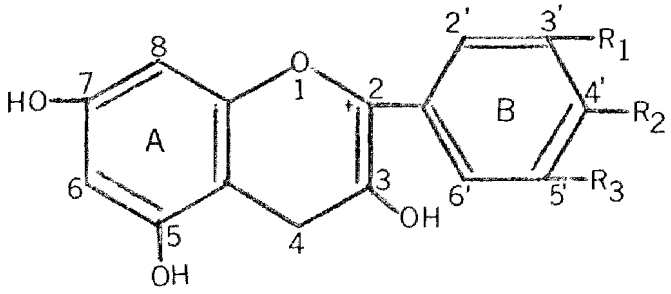
INTRODUCTION AND REVIEW OF LITERATURE

The variety of colors found in different organs of plants has presented a challenge to scientific investigators. Pigments are found in all plant organs--root, stem, leaf and flower. Are the pigments found concentrated in petals of the flowers the same pigments that are found in leaves and other organs of the plant? Are many pigments involved or a few which are modified in various ways? Questions such as these have led to the investigation of the pigments in plants.

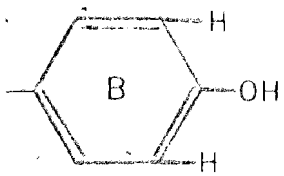
Plant pigments are divided into two groups: the water soluble flavonoids found in the vacuoles of the cell and lipid soluble ones located in the plastids (Arditti and Dunn, 1969). This investigation will deal with only one type of flavonoid, the anthocyanins. These pigments are responsible for the production of red, blue and purple coloring in the flowers. These pigments can be found in all organs of the plant and not just the flowers (Harborne, 1963a). Beside providing much permanent pigmentation in plants, anthocyanins appear transiently in young leaves and other organs in response to environmental change or sugar concentration (Harborne, 1967). During the late spring and summer it is not easy to determine the source of anthocyanins in leaves, since visual anthocyanin color is often masked by chlorophyll. The production of anthocyanins in the autumn is determined in part by heredity and in part by environmental factors

such as light and temperature. Low temperature commonly influence anthocyanin formation in the leaves of trees. Sugar accumulation due to the direct effects of light stimulate the synthesis of anthocyanins, as in the leaves of poison ivy in strong light in contrast to the yellow color of leaves growing in the shade. The tops of the turnip root, when exposed to light, forms anthocyanins. Phosphate deficiency in plants will stimulate the formation of anthocyanins in the leaves (Wilson, Loomis and Steeves, 1971). The purpose of this thesis study was to isolate and identify the anthocyanins in the leaves of Oxalis ortgiesii, Regel (Bailey, 1968).

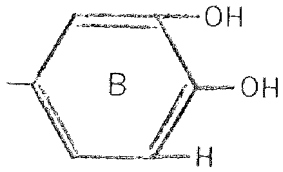
The anthocyanins are composed of glycosides (sugars) and a sugar-free portion called the aglycone or anthocyanidin. Six common anthocyanins and seven rare ones have been isolated and identified in plant material (Clevenger, 1964). The structure of the anthocyanins was established by Willstatter and later confirmed by Robinson to be based on the flavylium skeleton (Haysaki, 1962). The flavylium consist of two six-carbon benzene rings with a six-membered pyrylium ring structure between them (Figure 1). The chemical structure of the different anthocyanidins differ in arrangement of chemical groups attached to the flavylium skeleton (Figure 1). This may occur by hydroxylation or by hydroxylation and methylation (Haysaki, 1962). Some anthocyanins are also acylated with hydrocinnamic acids which



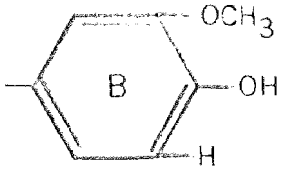
Flavylium Skeleton



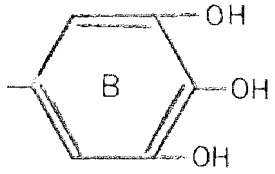
Pelargonidin



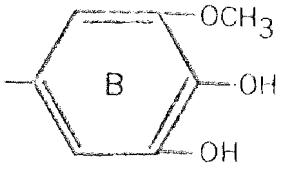
Cyanidin



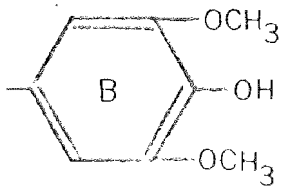
Peonidin



Delphinidin



Petunidin



Malvidin

Figure 1. Structure of flavylium skeleton and B ring structure of the common anthocyanidins.

are bonded through the hydroxyl groups of the sugars (Harborne, 1963a).

The flavonoids are useful as chemotaxonomic guides according to Bate-Smith (1963). His reason is this: these pigments are not actively concerned in cellular metabolic processes, therefore they are present in more or less constant amounts in the same tissues of the species that are grown under normal environmental conditions. The information man learns of the relationships between physiological functions and genetic make-up of the species, genera, families and other classes of plants will aid the taxonomists in his work. Alston and Hagen (1958) in their work with the genus Impatiens found that the pigments are controlled by different sets of genes. They proved that in a group of plants which have similar genetic make-up, the pigments will be similar. If so, their relationships can be determined through the identification of the synthesis which a particular species can or can not perform such as the forming of a particular type of pigment.

Lawrence et al. (1939) established evidence for an evolutionary trend for the anthocyanins. Cyanidin was considered to be the most primitive configuration, and gave rise to the more advanced pelargonidin, delphinidin, or methylated forms by one or more steps. The loss of steps was considered to be advancements by Bate-Smith (1963). This conflict was resolved by Harborne (1962), who stated

that the addition of steps have occurred as a result of natural selection, which counter-acted other mutations. Because the blue flower color is more attractive to insect pollinators than red, complexed blue anthocyanins were biosynthetically produced by some plants. The cyanidin-3-glycosides rarely produced blue color, but the more abundant co-pigmented, acylated, methylated, anthocyanin triglycosides did.

Harborne (1963a) summarized the frequency of the three common anthocyanidins. Cyanidin is the most common, being isolated in 80% of permanently pigmented leaves, 60% of fruits and 50% of flowers. Delphinidin was next in frequency followed by pelargonidin. Delphinidin is found in 90% of all blue flowers. Pelargonidin is found more frequently in cultivated plants than in wild ones. Malvidin, a methylated form of delphinidin is abundant in the families Geraniaceae, Malvaceae and Primulaceae. Petunidin is not widely distributed in wild plants due to the fact that methylation and addition of a third hydroxyl group in the anthocyanidin "B" ring appear to have evolved together. Few plants have the ability to methylate the hydroxyl groups in the 5- and 7-positions of the anthocyanidins; therefore, these methylated anthocyanidins are rare in the plant kingdom.

Lawrence et al. (1939) stated that anthocyanins found in the various organs of the plant may each have a different anthocyanidin. Leaves and fruits tend to have simpler

pigments than the flowers. Usually in the investigation of a species only the pigment of one organ is examined. The anthocyanidins may vary in a plant but the pattern of glycosidation is usually more consistent. If data is not available on the flower pigments which are mostly studied, the results obtained from fruits or leaves can be used, keeping in mind the anthocyanidin may be simpler in structure.

From the years 1913 to 1916, Willstatter and his collaborators pioneered in the isolation and identification of anthocyanins (Harborne, 1967). Robinson and Robinson (1931) continued with Willstatter's work in the pigment field. They were primarily interested in the pigments in flowers because of the ease of collecting petals, amount of pigment present in petals, and the extraction of the colored material was not hard. The Robinsons' isolation and identification of anthocyanins exemplified early chemotaxonomic work.

The research of Consden, Gorden and Martin (1944) with paper chromatography of amino acids served as a guide for other areas of research. Partridge (1946) applied paper chromatography to the analysis of reducing sugars. Bate-Smith (1948) introduced the use of paper chromatography to the investigation of the anthocyanins. He discovered the value of butanol:acetic acid:water (4:1:5) as a solvent which would give good separation of anthocyanins. He used

acid hydrolysis to cleave the anthocyanin molecule and identified the sugars by chromatography. He also introduced the procedure of direct application of the pigment extracts to chromatographic paper for purification. Bate-Smith (1949) concluded that anthocyanin structure can be related to Rf values.

Harborne (1958a) found that unknown pigments could be economically and quickly be identified by chromatographic techniques. He concluded that special care had to be used with butanol:acetic acid:water because its age would affect Rf values considerably. The solvent systems introduced by him are the standard solvent systems used today in anthocyanin investigations. Harborne found that structural modifications such as hydroxylation, methylation, glycosidation and acylation all will have characteristic effects on the Rf value of an anthocyanin.

Spectral analysis was also employed along with the chromatographic methods to investigate the structure of anthocyanins. Harborne (1958b) employed spectral examination to characterize anthocyanins. The spectral maxima were of value in finding the position of sugar attachment to an anthocyanidin. Jurd (1962) found that the main absorption region for anthocyanins is in the 500-550 nm region of the visible spectrum. The position of the visible maximum shows considerable shifts with changes in pH and solvent. The addition of glycosides to anthocyanidins shifts

its absorption maximum toward shorter wave lengths. The addition of sugar into the 3- or into the 3, 5- positions produces a 10-15 nm shift toward shorter wave lengths. Harborne (1958b) determined that anthocyanins having a free hydroxyl group in the 5- position show a distinct shoulder to the main absorption maximum in the 410-450 nm region.

Adding a few drops of $AlCl_3$ solution (5%, w/v) produces a 15-50 nm shift of the spectral maximum of anthocyanins that contain adjacent hydroxyl groups. Anthocyanins which do not contain an adjacent hydroxyl grouping will be unaffected by addition of $AlCl_3$. This reagent was introduced by Geissman, Jorgensen and Harborne in 1953 (Jurd, 1962). Swain (1954) indicated that the shift was due to the chelating effects of aluminum with the orthohydroxycarbonyl groups.

Harborne (1958b) reported that acylation of anthocyanins can be recognized by spectral analysis. Acylated anthocyanins will show two peaks in the u.v. region while simpler anthocyanins show a single peak in this region of the spectrum.

Chandler and Harper (1961) employed acid hydrolysis to identify the aglycone and sugars of the anthocyanins of the blackcurrant fruits. The sugar components of anthocyanins released after complete hydrolysis can be identified by use of paper chromatography. Absolute identification can be made of the anthocyanidin and the sugars produced by chromatographic and spectral properties according to Harborne (1958a, 1958b).

Alkaline hydrolysis is used to identify the organic acid of an acylated anthocyanin. Koeppen and Basson (1966) in their research of the pigments of Baclinka grapes employed alkaline hydrolysis to identify the acylated anthocyanin that was isolated.

Robinson and Robinson (1931, 1932) by using the series of methods they developed investigated the anthocyanins in many plant species. They reported that the leaves of four species of Maple trees contained cyanindin-3-glucoside. The same anthocyanin was reported by them in the red leaves of Ampelopsis hederacea (pepper vine). They found that the purple leaves of the Antirrhinum major (snapdragon) contained pelargonidin-3-pentoseglycoside.

Harborne and Sherratt (1957) employed paper chromatographic methods and acid hydrosis to identify the anthocyanins of the leaves of the copper beech, Fagus sylvatica. They reported the presences of pelargonidin-3-glucoside and trace amounts of cyanidin-3-galactoside.

DiGregario and DiPalma (1966) worked on the leaves of the Venus Flytrap because of the red pigment in the cells of the digestive glands, the inner layer of leaf cells and in cells at the base of the trigger hairs. The anthocyanin was identified by using paper chromatographic methods, acid hydrolysis and spectroscopic studies. The anthocyanin was determined to be cyanidin-3-glucoside.

Harborne (1963b) on the investigation of the leaves

of the Purple Cabbage reported finding cyanidin-5-glucoside-3-sophoroside acylated with sinapic acid. The same anthocyanin was isolated from the roots. Tanchev and Timberlake (1969a) isolated cyanidin-5 glucoside-3-sophorside from the leaves of the Red Cabbage. They also found the anthocyanin acylated with sinapic acids. Both the Red and Purple Cabbage are varieties of the same species.

Tanchev and Timberlake (1969b) investigated the purple leaves of Cotinus coggygria or commonly called the smoke-tree. The pigments isolated from the leaves contained delphinidin-3-galactoside, cyanidin-3-galactoside and petunidin-3-glucoside.

No research has been reported on the Oxalidaceae. Of the twenty-three families of the Germaniales, only four of the families have any anthocyanins isolated, identified and reported in the literature. The Oxalidaceae is one of the families found in the order Germaniales.

Robinson and Robinson (1932) reported from their investigative survey of anthocyanins, that three species of Pelargonium had the anthocyanin malvindin-3-5-diglycoside present. They reported another anthocyanin present but could not identify it. Harborne (1961) identified the unidentified pigment as pelargonidin-3-5-diglucoside. The Robinsons reported that the bracts of Euphorbia pulcherrina contained cyanidin-3-pentoseglycoside. Asen (1958) employed modern methods of chromatography and spectroscopy identified

several anthocyanins present in the bracts. He identified four different anthocyanins during his research. They were cyanidin-3-glucoside, cyanidin-3-rhamnoglucoside, pelargonidin-3-glucoside and pelargonidin-3-rhamnoglucoside. The Robinsons also isolated the anthocyanin pelargonidin-3-bioside from the flowers of the Nasturtium or Tropaeolum major. Harborne (1963b) employed chromatographic and spectrophotometic methods which are in common use today to identify the anthocyanin of the Nasturtium as pelargonidin-3-sophorside. Chandler (1958) investigated the fleshy endocarp of the bloodorange Citrus sinensis for anthocyanins. By the use of paper chromatographic methods and acid hydrolysis he identified the major pigment as cyanidin-3-glucoside and the minor pigment as delphinidin-3-glucoside.

MATERIALS AND METHODS

The Oxalis ortgiessi used in this study was grown in the greenhouse at Drake University and 250 gms. of leaves collected for extraction.

Leaf pigments were extracted in 1% HCl-methanol solution (v/v) overnight in the cold. The chlorophyll was removed from the crude extract by washing with petroleum ether until the petroleum ether layer was colorless. The crude extract was concentrated by evaporation in vacuo at less than 50° C in a rotary flash evaporator. The crude

concentrated extract was streaked in a 1 cm band with a pipette on Whatman No. 3 chromatography paper, and the chromatogram developed by ascending chromatography for 3 hours using solvent b (Table 1). The more soluble pigments are carried closer to the solvent front and the less soluble lag behind. The chromatogram was dried and the single visable pigment band cut out and eluted from the paper with 0.01% methanolic HCl. An absorption spectra was obtained on the purified pigment using a Beckman model DB Spectrophotometer with synchronized log recorder. The shape of the absorption spectra curve and the wave length at which maximum absorption occurs can be used to characterize anthocyanins (Harborne, 1958b).

The volume of the purified pigment was reduced again by evaporation in vacuo at less than 50° C. The concentrated pigment was spotted on 22 by 22 cm Whatman No. 1 chromatography paper and developed using various solvent systems (Table 1).

The chromatograms were run using solvents a, b, c and d (Table 1) and the Rf values were determined after drying (Table 2). The chromatograms were examined in ultra-violet light and any fluorescing pigment spots were marked and color noted. The chromatograms were also exposed to ammonia fumes. Different anthocyanins will react differently when seen in visible light and characteristically change color on fuming with ammonia. Examination under u.v. light is also of value,

Table 1. Chromatographic solvent systems

Abbreviation		Composition	Volume Ratio	Layer Used
a	BAW	n-butanol:acetic acid:water	4:1:5	Top
b	1% HCl	12N hydrochloric acid:water	3:97	Miscible
c	BuHCl	n-butanol:2N hydrochloric acid	1:1	Top*
d	HAc:HCl	acetic acid:12N hydrochloric acid:water	15:3:82	Miscible
e	EtA-Py	ethyl acetate:pyridine:water	12:5:4	Miscible

* BuHCl aged 24 hours before use and paper equilibrated in lower aqueous phase of BuHCl for 24 hours.

since differences in ability to fluorescence provide a means to their identification (Harborne and Sherratt, 1957).

A spectral absorption curve was determined on the purified pigment after the addition of 5 drops of ethanolic solution of $AlCl_3$ (5%, w/v) to aid in the identification of the anthocyanin. By using ethanolic $AlCl_3$ the point of maximum absorption may be shifted in some anthocyanins and these changes give definite information about the structure of the anthocyanins (Harborne, 1958b; Jurd, 1962).

The concentrated pigment was hydrolyzed to determine the anthocyanidin and carbohydrate(s) that make up the anthocyanin. Two methods of acid hydrolysis was used to determine the glycosides and anthocyanidin present in the pigment. In the first, 3 ml of concentrated extract was refluxed using a "cold finger" with 10 ml of 2N HCl in ethanol for 30 minutes (Harborne, 1962). The 2N HCl was replaced with 0.5N HCl in the second method and hydrolysis carried out in the same manner. The hydrolysates were reduced in volume by evaporation <u>in vacuo</u> at less than 50° C. The concentrated hydrolysates were spotted with a micropipette on 22 by 22 cm Whatman No. 1 chromatography paper. Glucose, galactose and rhamnose were spotted as markers. The ascending chromatograms were developed using solvents a and e (Table 1). The Rf value was taken from the chromatograms for the aglycone spot. The chromatograms were sprayed with aniline hydrogen phthalate (Chandler and Harper, 1961). The glycosides are

identified by comparison with the marker spots. The aglycone was eluated in 0.01% Hcl:methanol and a spectral curve taken on the Beckman DB Spectrophotometer.

Alkaline hydrolysis was done on the pigment extract. The extract (1 ml) was hydrolysis in aqueous 2N NaOH (1 ml) for 3 hours at 20° C. The hydrolysate was acidified with 0.3 ml of concentrated HCl and divided in equal amounts. One-half was extracted with 3 ml of amyl-alcohol and the other half with diethyl ether. Chromatograms were run using solvents a and e (Table 1). Ferulic, caffeic and p-coumaric acids were used as markers (Koeppen and Basson, 1966). The chromatograms were dried and then fumed with ammonia and examined under u.v. light.

RESULTS

During purification of the crude leaf pigment of Oxalis, the chromatogram developed in 1% HCl gave only a distinct band.

The purified leaf pigment's Rf values (Table 2) was obtained by averaging the Rf values from 18 different spots from the chromatograms of each solvent. The Rf of 0.36 from BAW and the Rf of 0.20 from 1% HCl did not vary more than a ± 0.01 from the mean Rf for that solvent. The Rf of 0.26 in Bu:HCl and the Rf of 0.61 in HAc:HCl did not vary more than ± 0.02 from the mean Rf for that solvent.

Table 2. Rf value of leaf pigment of Oxalis ortgiessi.

Solvent	Rf
BAW	0.36
BuHCl	0.26
1% HCl	0.20
HAc:HCl	0.61

Table 3. Rf values and spectral maxima of the anthocyanidin from the leaves of Oxalis ortgiessi.

Rf in Solvents		Spectral Maxima 0.01% HCl:Methanol	
BAW	EtHAc:Pyr		
		275 nm	542 nm
0.58	0.49		

When the chromatogram was examined in visible light, the pigment was mauve. Exposing the chromatogram to ammonia fumes caused the pigment to turn blue. In u.v. light, the pigment spots fluoresed cerise.

The spectral measurement, determined in 0.01% HCl: methanol, gave maxia at 534 nm, 328 nm and 282 nm. The spectral maxima was not changed by the addition of a few drops of $AlCl_3$ solution.

The components of acid hydrolysis of the anthocyanin were examined chromatographically. Glucose and rhamnose were identified by comparison with known sugars treated in the same manner.

The anthocyanidin was identified by its Rf values and the absorption maxima. These values are presented in Table 3.

Ferulic acid (3-methoxy-4-hydroxy-cinnamic acid) was identified after alkaline hydrolysis of the pigment. A spot corresponding to ferulic acid was found where the known cinnamic acid and its derivitives were used as markers on a chromatogram.

DISCUSSION

Basically, anthocyanins are identified by observing their color in solution or on chromatography paper, their Rf values and spectral properties. These methods were used

to identify the anthocyanin isolated from leaves of Oxalis ortgiessi.

The crude pigment separated into only a single band during purification in 1% HCl and HAc:HCl. This was not true in BuOH:2N HCl. Two spots were observed in this solvent. The main spot had a high Rf and the minor spot had a low Rf value. The main spot was eluted and concentrated in vacuo at less than 50° C and rechromatogramed in BuOH:2N HCl with the same results. Harborne (1958a) regarded this to be the main chromatographic characteristic of acylated anthocyanins.

The spectral absorption curve did not show a shoulder in the 410-450 nm region. This indicated there was a glycosidic linkage at the 5-OH position (Harborne, 1958b). When a few drops of $AlCl_3$ were added, there was no evident shift in the spectral maximum from 534 nm and this is what would be expected in anthocyanins where there is only one hydroxyl group free in the B ring (see fig. 1) in the 4' position (Harborne, 1958b). There was two peaks in the u.v. region of the spectrum. One peak was located at 328 nm and the other at 282 nm. This was due to the superimposition of the absorption of a type of Cinnamic acid upon that of anthocyanin absorption. This implies the presence of a hydroxyl aromatic acid as an acyl component (Harborne, 1958b). The spectral maxima obtained in this investigation of 534 nm, 328 nm and 282 nm corresponds to those reported by Harborne (1960) for

malvidin-3-rhamnoglucoside-5-glucoside acylated with ferulic acid (4-hydroy-3-methoxycinnamic acid).

The observation of the color of the purified pigment in visible light, u.v. light and after being exposed to ammonia fumes aided in the identification of the pigment. The mauve color in visible light and the blue color obtained after exposure to ammonia fumes are characteristic for malvidin triglycosides (Bate-Smith, 1948). The fluorescent cerise color shown by the pigment spot in u.v. light is indicative for malvidin-3-4hamnoglycoside-5-glucoside (Harborne, 1958a).

The sugars that were examined after acid hydrolysis of the anthocyanin also agreed with the above since glucose and rhamnose were isolated and identified chromatographically by comparison with known sugars. The anthocyanidin was identified as malvidin by the Rf value of 0.58 obtained in BAW and the spectral maxima of 542 nm and 275 nm which are identical to those listed in the literature (Anditti and Dunn, 1969).

After alkaline hydrolysis, ferulic acid was identified from the hydrolysate. The ferulic acid would be linked to the anthocyanin through the sugar in the 3-position and not to one of the free hydroxyl groups present in the anthocyanidin (Harborne, 1967).

The Rf values that were recorded during this investigation are in close agreement with those reported by Harborne

(1958a) for acylated malvidin-3-rhamnoglucoside-5-glucoside. Harborne (1967) stated that the Rf value is the most important single characteristic for the identification of an anthocyanin. The various glycosides of any one anthocyanin can be separated by means of their Rf values in four common solvent systems, because the movement of a pigment indicates clearly the nature and number of sugar and other substitutes it contains.

The isolation and identification of malvidin-3-rhamnoglucoside-5-glucoside acylated with ferulic acid from the leaves of Oxalis ortgeissi is the first reported for a species of the Oxalidaceae. Anthocyanins have been reported in the leaves of a number of plants and used in place of flower pigments to aid in taxonomic work. The study of the leaves of four maple species by Robinson and Robinson (1931 and 1932) would be an example of this type. The young leaves of other Oxalis species contain anthocyanin, but no work has been done on these plants. Because this species of Oxalis was developed as an ornamental plant by Ortgies, a German horticulturist (Bailey, 1968), it would not seem to be of much taxonomic value for the Oxalidaceae.

SUMMARY

The purpose of this investigation was to isolate and identify the anthocyanin in the leaves of Oxalis ortgiessi. This purpose was achieved to the extent that an anthocyanin was isolated and identified.

In this study, leaf extracts were purified by paper chromatography, eluted from chromatograms and studied chromatographically and spectrally to determine the detailed structure of the anthocyanin. The hydrolyzed sugars and acid were isolated and identified. Observation of color in visible light, u.v. light and after exposure to ammonia fumes were used to aid in the identification. Malvidin-3-rhamno-glucoside-5-glucoside acylated with ferulic acid was identified by these procedures.

This investigation suggests that the following research is needed. Isolation and identification of the pigments in the flowers of the species should be studied to see if there is any relationships between the leaf and flower pigments. Pigment identification needs to be done on the young leaves and flowers of other species of the Oxalidaceae so that some taxonomic relationships may be shown within the family and with other families in the order Geraniales.

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